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(57) Abstract

The invention provides a means for identifying novel antibiotics by screening for compounds that inhibit bacterial ribosome assembly and ribosomal protein synthesis, which comprises the screening of test compounds to identify those that inhibit the activity of a bacterial ribosomal protein. The *in vitro* and *in vivo* assays detect (a) increased translation of a reporter mRNA that is normally repressed in the presence of a ribosomal protein, (b) increased growth in cells that over-express ribosomal protein, and (c) decreased binding of the ribosomal protein to its target RNA. The invention encompasses the (a) methods of screening and testing compounds by the above methods, (b) compounds that are identified in the assay which inhibit ribosome assembly and protein synthesis and (c) methods for treatment using said compounds.

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METHODS AND COMPOSITIONS FOR IDENTIFICATION OF INHIBITORS OF RIBOSOME ASSEMBLY

1. INTRODUCTION

The present invention relates to methods for identifying novel antibiotics by screening for compounds that inhibit ribosome assembly in bacteria. The invention relates to targeted, efficient, and high-throughput screens to identify small molecules, compounds, peptides, etc. that interfere with RNA-protein interactions required for bacterial ribosomal protein synthesis and ribosome assembly. Such screens encompass both *in vivo* and *in vitro* assays. The invention further encompasses antibiotic candidate compounds identified using such screening methods.

2. BACKGROUND OF THE INVENTION

New antibiotics are desperately needed. The widespread use of antibiotics over the past half century has lead to the emergence of bacterial strains that are resistant to nearly all antibiotics now in use. Thus there is an immediate need to develop fast and efficient methods for producing new antibiotics to combat the increasing number of these antibiotic-resistant strains (Chopra et al., 1997, Antimicrob. Agents Chemother., 37:1563-1571; Cohen, 1992, Science, 257:1050-1055; Kunin, 1993, Ann. Intern. Med., 118:557-561; Neu, 1992, Science, 257:1064-1073; Tenover & Hughes, 1996, JAMA, 275:300-304). Ideally, new classes of antibiotics can be discovered that will be toxic to a broad range of pathogenic bacteria, and, at the same time, be harmless to their human hosts. It is also hoped that these new antibiotics will target cell components and processes other than those targeted by existing antibiotics, so that resistant strains would not already be immune to the new drugs.

Traditional approaches to antibiotic development have failed to meet these needs. One commonly used approach involves chemical modification of an existing antibiotic to produce a more potent formulation. Another approach involves screening for compounds that target the resistance mechanism of a known antibiotic. Such compounds are then be used in conjunction with the known antibiotic to improve its efficacy. These

Antibiotics that act against the protein biosynthetic machinery, *e.g.*, tetracyclines, chloramphenicol, erythromycin and streptomycin, are valuable in treating bacterial infections (Gale et al., 1981, The molecular basis of antibiotic action 2nd ed., John Wiley & Sons, London, United Kingdom; Russell & Chopra, 1996, Understanding antibacterial action and resistance, 2nd ed., Ellis Horwood, New York, NY). For example, erythromycin and other macrolide antibiotics are effective against both gram positive and gram negative bacteria. However, erythromycin-resistant strains of *Streptococcus* and *Staphylococcus* are known, and may be on the rise. The exact mechanism of action of macrolide antibiotics still is not completely understood, although it has been suggested that erythromycin inhibits a step in translation between initiation and elongation (1984, Omura S., in Macrolide Antibiotics, Orlando, FL, Academic Press).

One important step in protein biosynthesis is the translation of genetic information from messenger RNA (mRNA) to proteins, an essential cellular process for all living organisms, including infectious pathogens. Translation requires the coordinated interplay of mRNA, ribosomes, tRNAs, and a number of specialized proteins (Hill et al., 1990, The Ribosome. Structure, Function and Evolution. Washington, DC: Am. Soc. Microbiol.; Nierhaus et al., 1992, The Translational Apparatus. Structure, Function, Regulation, Evolution. New York: Plenum.; Matheson et al., 1995, Biochem. Cell Biol. 73:739-1227; Zimmermann et al., 1996, Ribosomal RNA. Structure, Evolution, Processing, and Function in Protein Biosynthesis. Boca Raton, FL: CRC).

The *E. coli* ribosome, which has been the subject of intense research for over 30 years, is composed of two large particles, the 30S and the 50S subunits. The 30S subunit consists of a 16S rRNA molecule and 21 small ribosomal proteins ("r-proteins"). The 50S subunit is composed of two ribosomal RNA (rRNA) molecules, 23S and 5S rRNA, and 31 large ribosomal proteins.

While prokaryotic ribosomes are similar, regardless of species, they differ in many respects from eukaryotic ribosomes. In general, mammalian ribosomes are more complex than their bacterial counterparts. Mammalian rRNAs are larger – 18S and 28S as compared to 16S and 23S for bacterial rRNAs. There are many fewer r-proteins found in *E. coli* ribosomes (52) than in eukaryotic ribosomes (70-82). Similarly, ribosomal proteins from different species of bacteria are closely related, but show little to no similarity to

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The invention provides *in vivo* assays to identify test compounds that interfere with the specific binding of an r-protein to an RNA binding site. The invention encompasses a method for screening for a test compound wherein the translation of a reporter mRNA in a test cell contacted with a test compound is compared to the translation of the reporter mRNA in a test cell not contacted with the test compound. An increase in translation in the cell contacted with a test compound indicates that the test compound inhibits the activity of the respective r-protein in the test cell. In one specific embodiment of this assay, a reporter gene in the test cell produces the reporter mRNA.

The invention further encompasses an *in vivo* assay to screen a test compound based on measuring the growth of a test cell comprising an excess of a specific ribosomal protein. An increase in growth of a test cell contacted with the test compound, relative to a test cell not contacted with the test compound, indicates that the test compound inhibits the activity of the ribosomal protein.

In specific embodiments, the *in vivo* assays utilize an *E. coli* test cell. In another specific embodiment, the ribosomal protein is S8. In another specific embodiment of the method, the ribosomal protein can be S1, S4, S7, S15, S20, L1, L4, L10, or L20. In yet another embodiment, the reporter gene used in the *in vivo* assays is luciferase, chloramphenicol acetyl transferase, green fluorescent protein, β-galactosidase, β-lactamase, or β-glucuronidase.

The invention also provides compositions to be used in the screening assays of the invention. The invention provides a test cell comprising the reporter gene construct and all the factors necessary for transcription and translation of the reporter gene.

The invention further provides *in vitro* assays. In an *in vitro* reaction, the reporter mRNA is incubated with an r-protein, a test compound and the factors necessary for translation of the reporter mRNA. A test compound is contacted with a reaction mixture comprising a bacterial repressor ribosomal protein and a reporter messenger RNA. The translation of a reporter mRNA is measured. An increase in the translation of the reporter mRNA in the presence of the test compound indicates that the test compound inhibits the activity of the repressor ribosomal protein.

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4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. The organization of ten ribosomal protein operons in *E. coli*. The repressor r-protein for each operon is underlined. The positions of their mRNA binding sites are indicated by double asterisks.

FIG. 2. The alignment of S8 amino acid sequences from a number of prokaryotic organisms.

FIG. 3. The sequence and secondary structure of S8 RNA binding sites in *E. coli* and *H. influenzae*. The similarity of the base pairing within the double-stranded regions of the conserved mRNA and rRNA stem-loop-stem secondary structures is shown.

FIG. 4. The sequence and secondary structure of the *E. coli* S8 RNA binding site and a hybrid S8 binding site analog (arrow indicate start condons). A. The stem secondary structure of the S8 mRNA binding site. B. The S8 16S rRNA binding site. C. A hybrid S8 RNA binding site analog. The S8 hybrid RNA binding site shown here is used in the example presented in Section 6.

FIG. 5. The native S8 mRNA regulatory region located within the *spc* operon. A. Schematic diagram of the primary sequence of the S8 mRNA regulatory region. The stop codon of L24, the Shine Delgarno sequence, and start codon of L5 are indicated in bold type. B. Sequence of the S8 binding site on the mRNA. S8 binds to these regions (underlined in part A) thereby preventing translation of the *spc* operon.

FIG. 6. The S8 mRNA reporter construct. A. Schematic representation of the construct used in the *in vivo* and *in vitro* translation assays. The construct includes a strong Shine-Delgarno sequence (SD), a mini open reading frame (orf), a second SD, the hybrid RNA binding site with initiator AUG, and the Renilla Luciferase reporter gene. B. The nucleotide sequence of the RNA binding region. The underlined sequence represents the sequence of the S8 RNA binding site. Details are given in the example presented in Section 6.

FIG. 7. *In vitro* translation assay. The amount of S8 protein added to *in vitro* translation assays is indicated along horizontal axis. Renilla luciferase units (RLU) are indicated on vertical axis. Details are found in the example presented in Section 6.

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assembly, a subset of ribosomal proteins, termed primary binding r-proteins, bind rRNA directly, and facilitate the binding of other ribosomal proteins.

Because the interaction of r-proteins with RNA is critical for proper ribosome function, and because proper ribosomal function is essential for the growth of bacterial pathogens, the inventors recognize that compounds which can interfere with the binding of ribosomal proteins to RNA are attractive candidates for antimicrobial drugs. In light of the high degree of conservation among ribosome components within the bacteria, targeting ribosome assembly will yield antibiotics with activity against a broad range of bacterial species. Moreover, such drugs will be highly specific against bacteria with little or no effect on mammalian ribosomes since it appears that there is little structural homology between the ribosome components of bacterial and eukaryotic organisms. Therefore, the interactions between ribosomal proteins (r-proteins) and their RNA binding sites are used as targets in the screening assays of the present invention.

RNA– protein interactions are important in a variety of cellular processes and are the subject of intense study. RNA molecules form complex secondary structures in solution (see, for example, Doudna, 1997, 388:830-1; Ramos et al., Curr. Opin. Struct. Biol., 1997, 7:317-23). In contrast to DNA, where protein–DNA recognition can often be predicted based on the primary sequence of the DNA molecule, the secondary structure of the RNA molecule can be more important than its primary sequence for protein recognition. RNA helices with internal loops or bulges typically occur in regions of RNA involved in protein interactions, suggesting that such structures are a common strategy for RNA-protein recognition (see, for example, Draper, 1989, Trends Biochem. Sci. 14:335-338).

In various aspects of the invention, compounds are assayed for their ability to interfere with the interaction of a ribosomal protein and an RNA target molecule. Such an RNA target comprises an RNA binding site for the ribosomal protein. The RNA binding site can be either the cognate binding site of the r-protein or a structurally analogous RNA, such that the interaction of the structurally analogous RNA with the r-protein is functionally similar to the interaction of the r-protein's cognate RNA binding site with the r-protein. Thus, a compound that interferes the interaction of a r-protein with the analog will also interfere with the r-protein's activity in its natural context. For example, an RNA binding site of the invention may comprise the sequence of the mRNA recognition site of a

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The reporter mRNA useful in the methods of the present invention may be produced by a variety of methods known in the art. The reporter mRNA can be produced by transcribing a DNA molecule, herein referred to as a reporter gene construct, which comprises a bacterial promoter operably linked to a reporter gene, wherein the reporter gene comprises DNA sequences corresponding to the RNA binding site for the respective r-protein.

The methods of the invention can be carried out *in vivo* in a test cell or in an *in vitro* reaction. A test cell of the invention comprises the reporter gene construct and all the factors necessary for transcription and translation of the reporter gene. The r-protein may be under the control of an inducible promoter to allow regulation of the amount of r-protein present in a test cell. In an *in vitro* reaction, the reporter mRNA is incubated with the r-protein, a test compound and the factors necessary for translation of the reporter mRNA. Alternatively, a reporter gene construct can be used, if the appropriate enzymes and factors are provided to produce the reporter mRNA *in vitro*. The factors necessary for transcription and/or translation may be supplied in the form of a cell extract. Purified r-proteins, translation factors, and transcription factors also can be used as a supplement to a cell extract. Accordingly, the invention also encompasses reporter mRNAs, reporter gene constructs, test cells comprising reporter mRNAs and/or reporter gene constructs, and reaction mixtures comprising reporter mRNAs and/or reporter gene constructs, useful in the various translation assays.

In yet another embodiment, the invention provides functional assays that evaluate the ability of a test compound to block the binding of an r-protein to an RNA binding site on an mRNA. The assays are based on measuring, in the presence of a test compound, the increase in growth of recombinant test cells that have been engineered to overexpress an r-protein. Normally, as a result of the overexpression, the recombinant cell exhibits a phenotype of retarded growth under a particular culture condition. In the presence of an effective test compound, the excess r-protein will be blocked from its binding site on the mRNA, resulting in a more rapid growth rate of the test cells. A test compound capable of interfering with the binding of an r-protein to its specific RNA binding site on an mRNA molecule can thereby be identified by an increase in the growth rate of the recombinant test cells.

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In another embodiment, to facilitate detection and quantitation, the binding assays of the invention employ components that comprise an affinity tag which allows a tagged component to be conveniently separated from the untagged components in a binding reaction. Typically, this can be accomplished by tagging one of the components, either the ribosomal protein or the RNA target molecule, with an affinity compound. The affinity tag can be captured by a binding partner of the affinity tag, which is immobilized onto a solid phase surface. After the binding reaction is complete, RNA-protein complexes comprising the affinity-tagged component, and excess unbound tagged component, can be captured onto the solid phase surface for measurement, by methods well known in the art.

Alternatively, an agent that specifically binds the ribosomal protein or the RNA target molecule may be used. Such an agent, *e.g.*, an antibody against the ribosomal protein, or a nucleotide sequence complementary to the RNA target molecule, can be immobilized on a solid phase surface to capture the products of the binding reaction. For example, the target RNA can comprise, in addition to the RNA binding site for a specific ribosomal protein, a nucleotide sequence complementary to the nucleotide sequence of another nucleic acid molecule which is immobilized onto a solid phase. The target RNA thus can be captured onto the solid phase by hybridization to the immobilized nucleic acid molecule. Use of antibodies against the ribosomal proteins or against nucleotide sequences complementary to the target RNA, provided that their interaction with their protein or RNA binding partner does not significantly alter the interaction between the r-protein and the RNA binding site, are within the scope of the invention.

The invention also encompasses RNA target molecules, gene constructs encoding the RNA targets, and recombinant cells containing the target RNAs and/or gene constructs encoding these RNAs. The invention further encompasses ribosomal proteins containing an affinity tag, gene constructs encoding ribosomal proteins containing an affinity tag, and recombinant cells comprising a gene construct encoding a ribosomal protein containing an affinity tag.

The compounds tested in the methods of the invention can be obtained from a wide variety of sources including collections of natural products in the form of bacterial, fungal, plant and animal extracts; and synthetic chemical libraries. Numerous means known in the art are available for the random, directed and combinatorial synthesis of a wide

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Amino acid sequences and nucleotide sequences of naturally occurring r-proteins are generally available in sequence databases, such as GenBank. Computer programs, such as Entrez, can be used to browse the database, and retrieve any amino acid sequence and genetic sequence data of interest by accession number. These databases can also be searched to identify sequences with various degrees of similarities to a query sequence using programs, such as FASTA and BLAST, which rank the similar sequences by alignment scores and statistics. Nucleic acids encoding the r-proteins and fragments thereof are provided, as well as nucleic acids complementary to and capable of hybridizing to such nucleic acids. The complete nucleotide sequences of many r-proteins and rRNAs are known in the art. Non-limiting examples of such sequences are those of *E. coli*, *Helicobacter pylori*, *Haemophilus influenzae*, *Mycoplasma genitalium*, and *Chlamydia trachomatis* (Blattner et al., 1997, *supra*; Tomb et al., 1997, *supra*; Fleischmann et al., 1995, Science, 269:496-512; Fraser et al., 1995, Science 270:397-403; Stephens et al., Science, Oct. 23, 1998).

Although the ribosomal proteins of *E. coli* are used herein for purpose of description of the invention, it is contemplated that the invention encompasses the use of equivalent ribosomal proteins of other bacteria. The *E. coli* ribosomal proteins and their equivalents in other bacteria are expected to share a high degree of sequence and structural similarity. Such equivalent ribosomal proteins, and their genes in other bacteria can be identified by techniques commonly known in the art. Non-limiting examples of such techniques include polymerase chain reaction using known or degenerate sequences as primers, immunoassays using cross-reacting antibodies, or hybridization assays using the *E. coli* gene as a probe.

The DNA encoding an r-protein of *E. coli* or another bacteria, herein referred to as an r-protein gene, may be obtained by standard procedures known in the art, such as by DNA amplification from DNA prepared from cells or from cloned DNA (e.g., a DNA "library"). Likewise, genes encoding ribosomal RNA from *E. coli* and other bacteria, herein termed rRNA genes, can also be obtained by such methods. Whatever the source, the r-protein gene or rRNA gene should be molecularly cloned into a suitable vector for propagation of the gene.

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Other methods for isolating the r-protein genomic DNA include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or making cDNA to the mRNA that encodes the r-protein. For example, RNA for cDNA cloning of the r-protein gene can be isolated from cells which express the regulatory r-protein. A cDNA library may be generated by methods known in the art and screened by methods, such as those disclosed for screening a genomic DNA library. If an antibody to the r-protein is available, the r-protein may be identified by binding of labeled antibody to clones that synthesize the putatively regulatory r-protein.

Other specific embodiments for the cloning of a nucleotide sequence encoding a r-protein, are presented as examples but not by way of limitation, as follows:

In a specific embodiment, nucleotide sequences encoding r-protein of another species of bacteria can be identified and obtained by hybridization with a probe comprising nucleotide sequence encoding the *E. coli* r-protein under conditions of low to medium stringency.

By way of example and not limitation, procedures using such conditions of low stringency are as follows (see also Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. USA 78:6789-6792). Filters containing DNA are pretreated for 6 h at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 X 10^6 cpm ^{32}P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 h at 40°C, and then washed for 1.5 h at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 h at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and reexposed to film. Other conditions of low stringency which may be used are well known in the art (e.g., as employed for identifying genes within the same family).

The r-protein or rRNA gene can be inserted into an appropriate cloning vector and introduced into host cells so that many copies of the gene sequence are generated. A large number of vector-host systems known in the art may be used such as,

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the ribosomal protein to the ribosomal binding site, by altering the conformation of the mRNA such that the ribosomal binding region is inaccessible by the ribosome, or by interfering with a subsequent stage of the translation process.

The organization of the *E. coli* the r-protein genes and other genes within each operon is shown in Figure 1. The ten translation repressor r-proteins (underlined in Figure 1) and their mRNA recognition sites (double asterisks in Figure 1) are discussed in detail herein. The nucleotide positions of the mRNA binding sites provided herein for each repressor r-protein corresponds to the numbering scheme used in the specific references cited, which are each incorporated herein in their entireties.

S8 is one of the most preferred target ribosomal protein for use in the methods of the invention. S8 is the fifth gene product of the *spc* operon, which encodes 11 r-protein genes (Ceretti et al., 1983, Nucl. Acids Res. 11:2599-2616; GenBank Accession No. X01563). As shown in Figure 2, ribosomal proteins equivalent to S8 in other bacteria are also known in the art, and share a high degree of sequence similarity with each other. The use of such equivalents of S8 in screening assays, and similarly, for equivalents of other repressor r-proteins are contemplated. S8 regulates translation of its mRNA by binding to a site at the beginning of the third gene of the operon, L5, between residues +22 to +81 within the double-stranded stem of a conserved stem-loop secondary structure (Gregory et al., 1988, J. Mol. Biol. 204:295-307). Figure 3 shows the secondary structure of the S8 binding site on the *spc* mRNA of *E. coli* and *H. influenzae*, and their similarities to the secondary structure of its binding site on 16S rRNA, as discussed in Section 5.1.2, *infra*.

The L4 protein (GenBank Accession No. X02613; Zurawski & Zurawski, 1985, *supra*), the product of the fourth gene of the S10 operon, regulates both the transcription and the translation of the S10 operon by binding to a site on the S10 leader sequence of the mRNA. Two adjacent hairpin loop structures formed by RNA base pairing of residues located between positions 84 and 145 (named HE) and 146 and 192 (named HG) are required for L4-mediated translational control (Zengel & Lindahl, 1994, *supra*). According to structure probing studies, L4 binds to a single-stranded region at approximately position 145 between the two hairpin loop structures HE and HG. The S10 leader sequences of several other enterobacteria have also been shown to be highly

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The L20 protein is part of a complex transcription unit with genes for L35 and two translation factors (Fayat et al., 1983, J. Mol. Biol. 171:239-261; GenBank Accession No. M10423). L20 is the repressor of its own gene and the gene for L35 (Lesage et al., 1992, J. Mol. Biol., 228:366). A region of the upstream *infC* gene, which encodes initiation factor-3, is known to be essential (Lasage et al., 1990, J. Mol. Biol., 213:465).

The S20 protein (Mackie, 1981, J. Biol. Chem. 256:8177-8182; GenBank Accession No. X04382) is encoded by a monocistronic operon and regulates its own translation. S20 regulation is thought to occur by binding of S20 to the 30S::S20-mRNA tertiary complex (Parsons et al., 1988, J. Bacteriol. 10: 2485) or by a mechanism involving repression ribosomes containing S20 (Gotz et al.,1990, Biochem. Biophys. Acta 1050:93).

S1 protein, the only translational regulatory protein that is not a primary binding protein, is the only protein encoded by the S1 operon.. The S1 protein has a general and weak affinity for RNA, particularly oligo(U)-containing sequences (Boni et al., 1991, Nuc. Acids Res. 19:155).

Due to the degeneracy of the genetic code, the term "ribosomal protein gene sequence" refers not only to the naturally occurring nucleotide sequence but also encompasses all the other degenerate DNA sequences that encode the ribosomal protein. Any technique for mutagenesis known in the art can be used to modify individual nucleotides in a DNA sequence, for purpose of making amino acid substitution(s) in the expressed peptide sequence, for creating/deleting restriction sites, or for adding affinity tags. Such techniques include but are not limited to, chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem 253:6551), oligonucleotide-directed mutagenesis (Smith, 1985, Ann. Rev. Genet. 19:423-463; Hill et al., 1987, Methods Enzymol. 155:558-568), PCR-based overlap extension (Ho et al., 1989, Gene 77:51-59), PCR-based megaprimer mutagenesis (Sarkar et al., 1990, Biotechniques, 8:404-407), etc. Modifications can be confirmed by DNA sequencing.

In addition, modified regulatory r-proteins can be synthesized comprising a peptide tag which facilitates recovery and purification. The peptide tag can be associated with any portion of the repressor r-protein, so long as such association does not alter the binding of the r-protein with the RNA binding site. In various embodiments, such a fusion

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A primary binding r-protein, or a peptide fragment comprising an RNA binding site thereof, can be obtained by purification of an r-protein or fragment from native sources, such as bacterial extracts. For example, ribosomal proteins, and fragments thereof, can be isolated from total extracts of cellular proteins, by methods well known in the art (Ausubel, *supra*). Alternatively, an r-protein or fragment may be obtained by molecular cloning and expression of ribosomal protein genes. Various methods can be used to obtain the coding region of such ribosomal protein gene sequences, including, but not limited to, DNA cloning, DNA amplification, and synthetic methods, as will be appreciated by those skilled in the art.

The nucleotide sequences of non-limiting examples of *E. coli* ribosomal protein genes that can be expressed by methods of the invention are published as follows: S4, GenBank Accession No. X02543 (Bedwell et al., 1985, Nucl. Acids Res. 13:3891-3903); S7, GenBank Accession No. ES3149 (Post & Nomura, 1980, J. Biol. Chem. 255:4660-4666); S8, GenBank Accession No. X01563 (Ceretti et al., 1983, Nucl. Acids Res. 11:2599-2616); S15: GenBank Accession No. J02638 (Regnier et al., 1987, J. Biol. Chem. 262:63-68); S17, GenBank Accession No. X02613 (Zurawski & Zurawski, 1985, Nucl. Acids Res.13:4521-4526); S20, GenBank Accession No. X04382 (Mackie, 1981, J. Biol. Chem. 256:8177-8182); L1: GenBank Accession No. J01678 (Post et al., 1979, Proc. Natl. Acad. Sci. U.S.A. 76:1697-1701); L2, GenBank Accession No. X02613 (Zurawski & Zurawski, 1985, Nucl. Acids Res. 13:4521-4526); L3, GenBank Accession No. X02613 (Zurawski & Zurawski, 1985, *supra*); L4, GenBank Accession No. X02613 (Zurawski & Zurawski, 1985, *supra*); L7/12, GenBank Accession No. J01678 (Post et al., 1979, *supra*); L10, GenBank Accession No. J01678 (Post et al., 1979, *supra*); L11, GenBank Accession No. J01678 (Post et al., 1979, *supra*); L15, GenBank Accession No. X01563 (Ceretti et al., 1983, Nucl. Acids Res. 11:2599-2616); L20, GenBank Accession No. M10423 (Fayat et al., 1983, J. Mol. Biol. 171:239-261); L23, GenBank Accession No. X02613 (Zurawski & Zurawski, 1985, Nucl. Acids Res. 13:4521-4526); and L24, GenBank Accession No. X01563 (Ceretti et al., 1983, Nucl. Acids Res. 11:2599-2616). Due to the degeneracy of the genetic code, the term "ribosomal protein gene sequence" refers not only to the naturally occurring nucleotide sequence but also encompasses all the other degenerate DNA sequences that encode the ribosomal protein.

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(reporter mRNA), in the presence of a test compound, and determining the expression of the product encoded by the reporter mRNA .

The *in vivo* translation assay of the invention involves a test cell which comprises a reporter gene construct and all the factors necessary for transcription and translation of the reporter gene. Detailed description of reporter gene constructs and test cells of the invention are described in Section 5.2.1 and 5.2.2, respectively.

The *in vitro* assays of the invention are carried out using a reporter mRNA, an r-protein, a test compound, and other factors necessary for translation of the reporter mRNA. Such factors necessary for transcription and/or translation may be supplied in the form of a cell extract.

The *in vivo* assays and *in vitro* assays have their advantages and disadvantages. Generally, *in vitro* assays are more expensive since active cell extracts containing labile ingredients are necessary. However, it might be advantageous to use an *in vitro* assay where a compound is so potent as to be lethal to the host cell in an *in vivo* assay. Such compounds can be readily tested *in vitro*. It might also be desirable to carry out the assays *in vitro* if methods for manipulating genetic material in the target microbial organism are not well developed, or if it is hazardous to use pathogenic bacteria as test cells.

5.2.1 Reporter Messenger RNA

The term "reporter messenger RNA" or "reporter mRNA" as used herein refers to an RNA molecule comprising a specific RNA binding site for a ribosomal protein which is functionally associated with any ribonucleotide sequence that encodes a detectable polypeptide. When used in an *in vivo* assay, the reporter molecule, *i.e.*, the translation product of the reporter mRNA, should be easily detectable and distinguishable from other proteins present in test cells. Preferably, the reporter mRNA encodes a protein that is readily detectable either by its presence, or by its activity that results in the generation of a detectable signal. A reporter mRNA is used in the invention to monitor and report the ability of a test compound to block or interfere with the interaction of an r-protein with its RNA binding site. The term "functionally associated" as used herein refers to an arrangement wherein the ribonucleotide residues to which a specific r-protein binds and the coding region of a detectable polypeptide are joined and positioned such that the translation

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sequences required for transcription of the reporter gene, and/or sequences for maintenance of the construct in a test cell. The term encompasses a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of reporter gene sequences. Such reporter gene constructs of the invention are preferably plasmids. Hence, a reporter gene construct may comprise a promoter, one or more origins of replication, and one or more selectable markers which allow phenotypic selection of recombinant cells. The reporter gene construct may also provide unique or conveniently located restriction sites to allow severing and/or rearranging portions of the DNA inserts in a reporter gene construct. Typically, a reporter gene construct is created in the course of constructing the fusion of an r-protein binding site and the coding region of the reporter. A reporter gene construct is used in the invention to generate large amounts of reporter mRNA in test cells or *in vitro*. More than one type of reporter gene may be inserted into the construct such that the results may be assayed by different means. Standard molecular biology techniques can be used to construct reporter gene constructs of the invention.

The coding region of a reporter mRNA comprises a nucleotide sequence that encodes a reporter molecule which is capable of directly or indirectly generating a detectable signal. In many cases, the coding region of a reporter mRNA may also comprise a nucleotide sequence that is a part of the specific r-protein RNA binding site. Some of such nucleotide sequences may encode the amino terminal of a ribosomal protein the synthesis of which is under the control of the repressor r-protein.

Generally, although not necessarily, the reporter mRNA encodes a detectable protein that is not otherwise produced by the test cells. Many such detectable proteins and their coding regions have been described, and some are commercially available for the study of gene regulation. See, for example, Alam & Cook, 1990, Anal. Biochem. 188:245-254, the disclosure of which is incorporated herein by reference.

For convenience and efficiency, enzymatic reporters and light-emitting reporters are preferred for the screening assays of the invention. Accordingly, the invention encompasses histochemical, colorimetric and fluorometric assays.

A variety of bioluminescent, chemiluminescent and fluorescent proteins can be used as light-emitting reporters in the invention. A preferred reporter for use in the present methods is Renilla luciferase. Other sources of luciferase, an enzyme that requires

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generated or the remaining amount of substrate is related to the amount of enzyme activity. For some enzymes, such as β-galactosidase, β-glucuronidase and β-lactamase, fluorogenic substrates are available that allow the enzyme to covert such substrates into detectable fluorescent products (see, for example, U.S. Patent No. 5,070,012, and PCT Patent WO 96/30540).

Depending on the screening technique and nature of the signal used to assay the reporter gene expression, a reporter regimen can be used to aid directly or indirectly the generation of a detectable signal by a reporter molecule. A reporter regimen comprises compositions that enable and support signal generation by the reporter, such as substrates and cofactors for reporter molecules that are enzymes; e.g., X-gal, lactose-tetrazolium medium. Such compositions are well known in the art. Components of a reporter regimen may be supplied to the test cells during any step of the screening assay.

Any antigenic peptide or protein that can be detected by an antibody can also be used as a reporter, for example, growth hormone (Selden et al., Mol. Cell Biol., 6:3173). To facilitate detection by antibody binding in immunoassays, antigenic reporter molecules that are secreted or attached on the test cell surface are preferred.

5.2.2 Test Cells

Bacterial cells useful in the present invention may be obtained from private laboratory deposits, public culture collections such as the American Type Culture Collection, or from commercial suppliers. It is desirable to use bacteria that have been developed for drug screening processes, and for which conditions for growth, maintenance, and manipulations are known. The most preferred bacterial species that is useful as test cells is *Escherichia coli*. Other preferred bacterial species may include but not limited to *Bacillus subtilis*, and *Pseudomonas aeuroginosa*. *Escherichia coli* can be used as a model of many bacteria.

Test compounds that specifically interfere with the binding of an r-protein to mRNA in *E. coli* test cells are expected to have a similar inhibitory effect on the translation of the ribosomal RNA in other pathogenic species, such as but not limited to, *Vibrio* species, *Pseudomonas* species, *Acinetobacter* species, *Bordetella* species, *Campylobacter* species, *Haemophilus* species, *Neisseria* species and Enterobacteriaceae species, such as

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maintenance and growth of the test cell may be different from those for assaying candidate test compounds in the screening methods of the invention. Modified culture conditions and media are used to facilitate detection of the expression of a reporter molecule. Any techniques known in the art may be applied to establish the optimal conditions.

Test cell strains, cell cultures, cell lines generated by the above-described methods for the screening assays may be expanded, stored and retrieved by any techniques known in the art that is appropriate to the test cell. For example, the test cells of the invention can be preserved by stab culture, plate culture, or in glycerol suspensions and cryopreserved in a freezer (at -20°C to -100°C) or under liquid nitrogen (-176°C to -196°C).

To facilitate high throughput screening, the test cells can be cultured and assayed in an ordered array, such as multi-well plates. Typically, the individual cultures are inoculated and allowed to grow in the wells under the appropriate conditions. Manipulations of the cultures and fluid handling can be done with a multi-channel devices. Most of the transfers and manipulations can be automated and miniaturized, and performed by laboratory robots.

5.2.3 Test Cell Extracts

The term "test cell extract" as used herein refers to an extract of a bacterial cell that comprises at least the components of the translation system that is functionally capable of translating a messenger RNA with the appropriate signals *in vitro*. The appropriate signals include, at a minimum, the ribosome binding site (the Shine-Dalgarno sequence), and a suitably positioned initiation codon (about 8 to 14 bases from the ribosome binding site). The test cell extract may contain the target r-protein of the *in vitro* assay.

In various embodiments, the test cell extract of the invention is an extract of test cells of the invention as described above, which comprises components of a functional translation system as well as a functional transcription system. The reporter mRNA used in the *in vitro* assay is produced by the transcription system in the presence of a reporter gene construct.

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5.2.5 *In Vitro* Translation Assay

The *in vitro* translation assay of the invention is based on the same principle as the *in vivo* translation assays except that the interactions between the preselected ribosomal protein of interest and the test compound, and the translation process itself, are carried out not within a live cell, but in a reaction vial or microtiter plate, or any suitable container. Since the translation process is performed *in vitro*, all the necessary translation factors, cofactors, and tRNAs charged with amino acids must be provided. Such ingredients of the *in vitro* translation assays can easily be provided by using an extract of the appropriate bacterial cells. Depending on the ribosomal protein and the bacterial species of interest, it may be preferable to supplement the *in vitro* reaction with purified ingredients which can be obtained from native bacterial cells or from recombinant expression of the factor if available. In some cases, it may be advantageous to add an excess of the r-protein of interest to enhance the sensitivity of the assay.

In this assay, the r-protein is contacted with a test compound for a time period and under condition sufficient to allow the test compound to inhibit the activity of the ribosomal protein. The contacting of the r-protein with the test compound can be performed prior to mixing the ingredients of the translation reaction with the r-protein. Alternatively, the test compound and the r-protein can be added simultaneously to the translation extract. Standard conditions of buffer, temperature, etc, for *in vitro* translation can be used. Techniques known in the art to calibrate and optimize the reaction conditions may be applied. Any method known in the art for detecting or measuring translation of the reporter mRNA in vitro can be used, including methods for detecting or measuring signal generated by the reporter molecule.

5.2.6 Fast-growth Assay

In another embodiment, the invention provides a screening assay (the fast-growth assay) that uses a bacterial test cell that contains a nucleic acid sequence encoding a repressor ribosomal protein or a fragment thereof, operably linked to a promoter which is expressible in the cell such that the repressor ribosomal protein is produced in the cell.

The fast growth assay that is an assay is based on measuring, in the presence of a test compound, the increase in growth of recombinant test cells that have been

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promoter of ø10 for T7 RNA polymerase, or under control of this promoter plus the leader sequence of the T7 gene 10. These control sequences have been shown to be very efficient, and can easily be obtained and inserted in other plasmids, viruses or chromosomes to create a wide variety of other vectors and configurations for T7 RNA polymerase-directed expression of any repressor r-protein gene.

It is also useful if the amount of repressor r-protein present in a test cell can be regulated pursuant to the needs of the screening assay. For example, the promoter used in making the expression construct is an inducible promoter the transcriptional activity of which is responsive to the presence of an inducer molecule inside a test cell. Any inducible promoters known in the art, such as but not limited to, the lac promoter, can be used. Adjustment of the level of repressor r-protein in the cell can be accomplish through the addition of the lactose inducer, isopropyl-β-D-thiogalactopyranoside (IPTG).

5.3 Assays Based on Physical Binding

The methods of the present invention relate to *in vitro* screening assays for the identification of compounds that interfere with the binding of ribosomal proteins to RNA binding sites on a target RNA molecule. Such compounds may act as antagonists of ribosomal protein-RNA interactions in bacterial cells. Compounds identified by the binding assays described herein may therefore be novel antibiotic candidates, and can be analyzed further to test their effect on inhibiting bacterial cell growth in mammalian systems.

The ribosomal proteins that can be used in the present method comprise primary r-proteins and repressor r-proteins, as described in Sections 5.1.1 and 5.1.2, *supra*, or fragments thereof that comprise the RNA binding domain.

In the binding assays described herein, a bacterial r-protein is incubated with a target RNA molecule under conditions that allow RNA-protein interactions to occur, in the presence or the absence of a test compound. RNA-protein complexes are separated from the unbound RNA and/or protein component, and the complexes are then measured. A decrease in the formation of complexes in the presence of a test compound, relative to in its absence, identifies a candidate inhibitory compound.

Various methods may be used for separating RNA-protein complexes from the reaction. For example, in one embodiment, the RNA or protein component can be

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that can retain proteins or r-protein/RNA complexes, but not free RNA. The labeled RNA bound to the protein retained on the solid phase surface can then be measured in the presence of the test compound, and in a control reaction without the test compound. Alternatively, the labeled target RNA molecule passing through the solid phase surface can be measured. The amount of RNA retained on the support, relative to either the input RNA or the RNA that passed through the filter, reflects the relative amount of RNA-protein complexes formed in each case.

The assay can be used to measure the effect of a test compound on the affinity of an r-protein for its binding site on an RNA molecule. A compound that interferes with the binding of an RNA to an r-protein will result in fewer labeled RNA molecules retained on the filter in the presence of the test compound than in its absence. Thus, if a lower level of bound RNA is measured in the presence of a test compound relative to its absence, a candidate inhibitor of r-protein-RNA interaction is identified.

In one embodiment, the r-protein is first titrated with target RNA to determine the appropriate concentration for performing competitive filter binding assays. To do this, r-protein is serially diluted and mixed with RNA under conditions that promote specific RNA-protein interactions. Either the r-protein or the target RNA can be labeled. The mixture of RNA and protein is then filtered through a device to separate the RNA-protein complexes from one of the components, either the RNA or the r-protein. The specific RNA-protein complexes are measured. A concentration is chosen equivalent to the minimum amount of protein required to specifically bind the maximal amount, i.e., saturate, the target RNA. The binding assay is repeated in the presence of test compounds at the optimal concentration of r-protein determined from the initial binding assay.

In a specific embodiment, r-protein is serially diluted in physiological buffer, such as 50 mM Tris/acetate, pH 7.5, 20 mM magnesium acetate, 270 mM KCl, and diluted to give a concentration range of 0.1 to 10000 nM. Labeled RNA is renatured at 42°C for 15 minutes, cooled to 4°C, added to the r-protein, and incubate on ice for approximately 30 minutes. This binding mixture is then filtered over nitrocellulose membranes, by methods well known in the art. Non-specific retention of RNA can be measured by filtering the reaction mixture in the absence of r-protein. An concentration of r-protein is selected that

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Alternatively, the amount of labeled target RNA that is not associated with the support can be measured. This binding reaction is performed in the presence and in the absence of a test compound. The amount of labeled target RNA bound to the r-protein is compared.

Alternatively, the solid phase can comprise a partner molecule of an RNA molecule. In this case, the affinity tagged target RNA molecule, comprising a binding site for the r-protein, is incubated with a labeled r-protein under conditions that allow binding to occur. The target RNA or the tagged target RNA can be pre-attached to the solid phase comprising a binding partner. Alternatively, the binding reaction can be contacted with the solid phase subsequent to the incubation. The labeled r-protein binds to the target RNA and is captured by the solid phase. The unbound proteins can then be separated from the solid phase and the labeled r-proteins bound to the solid phase or remaining in solution can then be measured. The binding reaction is performed in the presence and in the absence of a test compound.

5.3.3 Chemical and Enzymatic Modification Assays

Chemical and enzymatic modifications can be used to probe RNA secondary structure and RNA-protein interactions before and after the introduction of an antibiotic candidate compound. These assays are powerful tools that can be used for screening candidate drugs, understanding their mechanism of action, and designing novel drugs by site-specific modification of drug candidates.

Modifying enzymes and endonucleases are useful for probing RNA secondary structure and RNA– protein interactions. Non-limiting examples of such enzymes include RNase T1, S1 nuclease, which can be used to monitor unpaired nucleotides, and RNase V1, which can be used to probe base-paired or stacked nucleotides. Chemical probes can also be used to modify bases at accessible positions. Such chemical probes include, but are not limited to, DMS (dimethylsulfate, which methylates A residues at the N-1 position, C at the N-3 position and G at the N-7 position), DEPC (diethylpyrocarbonate, which ethylates A residues at the N-7 position), and CMCT (1-cyclohexyl-3(2-(1-methylmorpholino)-ethyl) carbodiimide-p-toluenesulfonate, which modifies U residues at N-3).

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16S rRNA comprising the S8 recognition site is used, together with the S8 r-protein, in a binding assay. In a preferred embodiment, the RNA target molecule position comprising the central domain of the 16S rRNA molecule is used. Preferably, the central domain comprises RNA segments from about position 588 to about 606 and from about position 632 to about position 651 at the base of a hairpin (Gregory et al., 1984, J. Mol. Biol.178:287-302; Svensson et al., 1988, J. Mol. Biol. 200:301-308; Cerretti et al., 1988, J. Biol. Chem. 204:309; Gregory et al., 1988, 204:295-307). In another embodiment, the target RNA comprises residues from about positions 573 to about 865 of the 16S rRNA central domain.

In various embodiments, the polycistronic mRNA of an r-protein operon is used for screening for compounds that interfere with r-protein - RNA interactions. Such a polycistronic mRNA may encode, among other r-proteins, a repressor r-protein. As described above, the specific binding site for the repressor r-protein in several of the r-protein operons are known. Thus, it is not always necessary to use the entire polycistronic mRNA. In many cases, the repressor r-protein binding site on the mRNA is located in the leader sequence of a gene upstream of the gene that encodes the repressor r-protein. Some exemplary RNA binding sites for specific repressor r-proteins are provided herein.

In various preferred embodiments, the target RNA comprises a specific binding site for S8. In one specific preferred embodiment, a S8 RNA binding site analog is provided that comprises a hybrid of the mRNA and rRNA sequences, such that the secondary structure of the analog is functional equivalent and structurally similar to either the S8 mRNA binding site or S8 rRNA binding site, or both (see Figure 4). In another embodiment, the RNA binding site used in the binding assays is the mRNA binding site for S8 comprising the RNA segment between residues about +22 to about +81 of the *spc* operon (Gregory et al., 1988, J. Mol. Biol. 204:295-307). In another specific embodiment, the target RNA can comprise a larger segment of RNA from this region of the *spc* operon, comprising for example, residues +1 to about 150, +1 to about 300, or +1 to 1000 . Alternatively, the mRNA binding site for S8 can comprise the full-length polycistronic mRNA from the 5.9 Kb operon.

In another embodiment, the target RNA comprises an mRNA binding site for L4. In a specific embodiment, the mRNA binding site for L4 comprises the mRNA

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In yet another embodiment, the target RNA comprises an RNA derived from the L35 operon. In a specific embodiment, the target RNA comprises a region of the L35 leader sequence, and/or a region of the leader sequence of the *infC* gene (Lasage et al., 1990, J. Mol. Biol., 213:465).

In yet another embodiment, the target RNA comprises an RNA derived from the S20 monocistronic operon. (Mackie, 1981, J. Biol. Chem., 256:8177-8182; GenBank Accession No. X04382). In yet another embodiment, the target RNA comprises an RNA derived from the S1 operon and may comprises an oligo(U)-containing sequences (Boni et al., 1991, Nuc. Acids Res., 19:155).

5.3.5 Construction of Ribosomal Protein Partner Molecules

An r-protein useful in the present method can be an r-protein comprising an affinity peptide tag. In various embodiments, such an affinity tag can be a peptide fused to the ribosomal protein. Such a fusion protein can be made by ligating an r-protein gene sequence to the sequence encoding the peptide tag in the proper reading frame. A variety of peptide tags known in the art may be used in the modification of an r-protein, such as but not limited to the polyhistidine sequence (Petty, 1996, Metal-chelate affinity chromatography, in Current Protocols in Molecular Biology, Vol. 2, Ed. Ausubel et al., Greene Publish. Assoc. & Wiley Interscience), glutathione S-transferase (GST; Smith, 1993, Methods Mol. Cell Bio. 4:220-229), the *E. coli* maltose binding protein (Guan et al., 1987, Gene 67:21-30), and various cellulose binding domains (U.S. patent 5,496,934; 5,202,247; 5,137,819; Tomme et al., 1994, Protein Eng. 7:117-123), etc. Other peptide tags may impart fluorescent properties to an r-protein, e.g., portions of green fluorescent protein and the like. Other possible peptide tags are short amino acid sequences to which monoclonal antibodies are available, such as but not limited to the following well known examples, the FLAG epitope, the myc epitope at amino acids 408-439, the influenza virus hemagglutinin (HA) epitope. Other peptide tags are recognized by specific binding partners and thus facilitate isolation by affinity binding to the binding partner which can be immobilized onto a solid phase surface. The ribosomal protein gene product can be prepared using recombinant DNA techniques. For example, the ribosomal protein gene sequence can be introduced into a vector containing the sequence of a peptide tag, such that

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compound can be used to bind affinity-labeled or fusion proteins. In a specific embodiment, r-proteins are separated from a binding mixture comprising a GST-fusion r-protein and radioactively labeled mRNA by contacting such mixture with a glutathione-linked solid phase surface, such as glutathione sepharose beads. For example, the GST-fusion protein can be anchored to glutathione-agarose beads or a glutathione-sepharose column. A labeled target RNA comprising the ribosomal protein binding site can then be added in the presence or absence of the test compound in a manner that allows interaction and binding to occur. At the end of the reaction period, unbound material can be washed away. The interaction between the protein and the RNA can be detected by measuring the amount of radioactivity that remains associated with the glutathione-agarose beads. A compound that interferes with the binding of an RNA to an r-protein will result in fewer labeled RNA molecules retained on the beads in the presence of the test compound than in its absence. Therefore, if a lower level of bound RNA is measured in the presence of a test compound relative to its absence, a candidate inhibitor of r-protein-RNA interaction is identified.

In another embodiment, a fusion protein may be readily purified by utilizing an antibody specific for the fusion protein being expressed.

In yet another embodiment, an affinity-tagged ribosomal protein can be constructed by conjugation of an affinity compound to the ribosomal protein. Affinity compounds can be used, such as, but not limited to, biotin, photobiotin, or other compounds known in the art. In one embodiment, affinity compounds or affinity tags can be conjugated to the ribosomal protein through a polyfunctional crosslinker, and preferably a bifunctional molecule. As used herein, the term polyfunctional crosslinker encompasses molecules having more than one functional group that reacts with a functional group on the ribosomal protein. Typically, such crosslinker forms covalent bonds with an amino or sulfhydryl group on a polypeptide. For example, biotin N-hydroxysuccinimide esters may be used.

5.3.6 Labeling of Ribosomal Proteins and Target RNA Molecules

The target RNA molecule used in the physical binding assays described herein, comprises an RNA binding site for a ribosomal protein. Such an RNA binding site can be rRNA or an analog or fragment thereof. In addition, where the ribosomal protein is

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using chemiluminescent, colorimetric, or fluorescent substrates. In yet another embodiment, RNA can be labeled with biotinylated nucleotides, and detected using a streptavidin-linked detectable compound.

Ribosomal proteins may be labeled with a detectable marker, using methods for protein labeling known in the art. A "detectable marker" refers to a moiety, such as a radioactive isotope or group containing same, or nonisotopic labels, such as enzymes, biotin, avidin, streptavidin, digoxygenin, luminescent agents, dyes, haptens, and the like. Luminescent agents, depending upon the source of exciting energy, can be classified as radioluminescent, chemiluminescent, bioluminescent, and photoluminescent (including fluorescent and phosphorescent).

In one embodiment of the invention, the affinity capture assay may be performed using a scintillation proximity assay (SPA, Amersham). Either the RNA component or the r-protein component can be labeled with a biotinylated marker, and the other component can be labeled with a radioactive label such as ^{33}P. For example, an labeled RNA can be generated by standard *in vitro* transcription with the inclusion of a ^{33}P-labeled nucleotide triphosphate, for example, ^{33}P-CTP. The two components can be mixed together, under conditions that allow RNA -protein interactions to occur. The biotinylated r-protein and the labelled RNA-r-protein complexes can then be captured on streptavidin beads, and counted in a scintillation counter.

5.4 Structure – Function Analysis

Computer modeling and searching technologies permit identification of compounds, or the improvement of already identified compounds, that can inhibit ribosome assembly or disrupt ribosomal protein synthesis. Having identified such a compound or composition, the active sites or regions of a ribosomal protein or an RNA binding site can be identified using methods known in the art including, for example, from the amino acid sequences of peptides, from the nucleotide sequences of RNA, or from study of complexes of the test compound with ribosomal protein and/or RNA. In certain case, chemical or X-ray crystallographic methods can be used to find the binding site by finding where on the ribosomal protein and/or RNA the bound compound is found.

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compound to determine if an improved fit or interaction results. In this manner systematic variations in composition, such as by varying side groups, can be quickly evaluated to obtain inhibitory compounds of improved specificity or activity.

Further experimental and computer modeling methods useful to identify compounds based upon identification of the binding sites on ribosomal protein, and RNA, and related translation factors will be apparent to those of skill in the art. Of the approximately 52 r-proteins of *E. coli*, high resolution structures of nine have been determined, six from the large subunit, L7/L12, L6, L9, L14 and L1 (Leijonmarck & Liljas 1987, J. Mol. Biol. 195:555-580; Wilson et al., 1986, Proc. Natl. Acad. Sci. USA 83:7251-7255; Golden at al. 1993, EMBO J. 12:4901-4908; Hoffman et al. 1994, EMBO J. 13:205-212; Davies et al. 1996, Structure 4:55-66; Nikonov et al. 1996, EMBO J. 15:1350-1359), and three from the small subunit: S5, S6, S17 and S8 (Ramakrishnan & White 1992, Biochem. and Cell Biol. 73:979-986; Lindahl et al. 1994, EMBO J. 13:1249-1254; Jaishree et al., 1996, Biochemistry 35:2845-2853; Davies et al., 1996, Structure 9:1093-1104). The structural information on these ribosomal proteins can readily be adapted for purposes of modeling the binding of a test compound, and further database searching.

Examples of molecular modeling systems are the CHARMm and QUANTA programs (Polygen Corporation, Waltham, MA). CHARMm performs the energy minimization and molecular dynamics functions. QUANTA performs the construction, graphic modeling and analysis of molecular structure. QUANTA allows interactive construction, modification, visualization, and analysis of the behavior of molecules with each other.

A number of articles review computer modeling of drugs interactive with specific proteins, such as Rotivinen et al. (1988, Acta Pharmaceutical Fennica 97:159-166); Ripka (1988 New Scientist 54-57); McKinaly & Rossmann (1989, Annu. Rev. Pharmacol. Toxiciol. 29:111-122); Perry & Davies, OSAR: Quantitative Structure-Activity Relationships in Drug Design pp. 189-193 Alan R. Liss, Inc. 1989; Lewis & Dean (1989, Proc. R. Soc. Lond. 236:125-140 and 141-162); and, with respect to a model receptor for nucleic acid components, Askew, et al. (1989, J. Am. Chem. Soc. 111:1082-1090). Other computer programs that screen and graphically depict chemicals are available from companies such as BioDesign, Inc. (Pasadena, CA.), Allelix, Inc. (Mississauga, Ontario,

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partially or completely, physiological or biochemical parameters associated with or causative of a bacterial disease or condition.

5.5.1 Determination of MIC

The minimum inhibitory concentration (MIC) against bacterial organisms is determined for each test compound that is positive in the assay. Methods known in the art may be used such as broth microdilution testing, using a range of concentrations of each test compound (1993, National Committee for Clinical Laboratory Standards). Methods for Dilution Antimicrobial Susceptibility Tests For Bacteria That Grow Aerobically - Third Edition: Approved Standard, M7-A3). The MIC against a variety of pathogens are determined using the same method. Pathogenic species to be tested generally include: *E. coli*, *Enterococcus faecium*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus epidermis*, *Shigella flexneri*, and *Salmonella typhimurium*.

5.5.2 Cytotoxicity Testing

Unfortunately, toxicity does not always arise from the same mechanism of action as is responsible for growth inhibition in the targeted microorganism. Therefore, the selectivity of the target should not be assessed solely on the basis of these results.

Cytotoxicity can be measured by methods known in the art. One such method is assessing growth of mammalian cells in the presence of the test compound, using a protein binding dye, sulforhodamine B (SRB). SRB binds electrostatically to basic amino acids. Binding and solubilization of the dye can be controlled by changes in pH. SRB binds stoichiometrically to proteins in one pH range but can be solubilized and extracted for measurement in another. An increase in total protein is correlated to cell growth. Cell growth in the presence of compound is compared to growth without added compound to establish a growth inhibitory concentration (GI_{50}) (Skehan et al., 1990, J. Natl. Cancer. Inst., 82:1107-1112). Another method of measuring cytoxicity which may be used in an assay containing 3[4,5-dimethylthiazol-2-yl]-2,5,-diphenyltetrazolium bromide/2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt ("MTT/XTT") as

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silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well-known in the art.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The antibiotic compounds may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the antibiotic compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the antibiotic compounds may be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a

5.5.4 Administration

For administration to subjects, antibiotic compounds discovered by using the assays of the invention are formulated in pharmaceutically acceptable compositions. The compositions can be used alone or in combination with one another, or in combination with other therapeutic or diagnostic agents. These compositions can be utilized *in vivo*, ordinarily in a mammal, preferably in a human, or *in vitro*. In employing them *in vivo*, the compositions can be administered to the mammal in a variety of ways, including parenterally, intravenously, subcutaneously, intramuscularly, colonially, rectally, vaginally, nasally, orally, transdermally, topically, ocularly, or intraperitoneally.

As will be readily apparent to one skilled in the art, the magnitude of a therapeutic dose of an antibiotic compound in the acute or chronic management of an infectious disease will vary with the severity of the condition to be treated, the particular composition employed, and the route of administration. The dose, and perhaps dose frequency, will also vary according to the species of the animal, the age, body weight, condition and response of the individual subject. The determination of effective dosage levels, that is the dosage levels necessary to achieve the desired result, will be within the ambit of one skilled in the art.

Desirable blood levels may be maintained by a continuous infusion of an antibiotic compound as ascertained by plasma levels. It should be noted that the attending physician would know how to and when to terminate, interrupt or adjust therapy to lower dosage due to toxicity. Conversely, the attending physician would also know how to and when to adjust treatment to higher levels if the clinical response is not adequate (precluding toxic side effects).

Optionally, a second antibacterial compound may be used in combination with the compound identified by the method of the invention. The second antibacterial compound may be naturally occurring or synthetic. Suitable naturally occurring antibacterial compounds include, but are not limited to, aminoglycosides (including but not limited to dihydrostreptomycin, gentamycin, kanamycin, neomycin, paromycin and streptomycin); amphenicols (including but not limited to chloramphenicol); ansamycins (including but not limited to rifamycin); β-lactams such as carbapems (including but not limited to imipenem), cephalosporins (including but not limited to cefazedone and

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blood stream. Some examples of such specialized drug delivery vehicles which fall into this category are liposomes, hydrogels, cyclodextrins, and bioadhesive microspheres. These vehicles have been developed for chemotherapeutic agents.

Topical administration of agents is advantageous when localized concentration at the site of administration with minimal systemic adsorption is desired. This simplifies the delivery strategy of the agent to the disease site and reduces the extent of toxicological characterization. Furthermore, the amount of material to be administered is far less than that required for other administration routes.

Antibiotic agents may also be systemically administered. Systemic absorption refers to the accumulation of agents in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include: oral, intravenous, subcutaneous, intraperitoneal, intranasal, intrathecal and ocular. Each of these administration routes exposes the agent to an accessible target.

5.6 Target Infectious Agents

The antibiotic compounds identified by the methods of the infection can be used to treat infectious diseases in animals, including humans, companion animals (*e.g.*, dogs and cats), livestock animals (e.g., sheep, cattle, goats, pigs, and horses), laboratory animals (*e.g.*, mice, rats, and rabbits), and captive or wild animals.

Specifically, infectious diseases caused by bacteria including but not limited to, gram positive cocci, such as Staphylococci (e.g., *S. aureus*), Streptococci (e.g., *S. pneumoniae, S. pyrogens, S. faecalis, S. viridans*); gram positive bacilli, such as Bacillus (e.g., *B. anthracis*), Corynebacterium (e.g., *C. diphtheriae*), Listeria (e.g., *L. monocytogenes*); gram negative cocci, such as Neisseria (e.g., *N. gonorrhoeae, N. Meningitidis*); gram negative bacilli, such as Haemophilus (e.g. *H. influenzae*), Pasteurella (e.g., *P. multocida*), Proteus (e.g., *P. mirabilis*), Salmonella (e.g., *S. typhi murium*), Shigella species, Escherichia (e.g., *E. coli*), Klebsiella (e.g., *K. pneumoniae*), Serratia (e.g. *S. marcescens*), Yersinia (e.g., *Y. pestis*), Providencia species, Enterobacter species, Bacteroides (e.g., *fragilis*), Acinetobacter species, Campylobacter (e.g., *C. jejuni*), Pseudomonas (e.g., *P. aeruginosa*), Bordetella (e.g., *B. pertussis*), Brucella species, Fracisella (e.g., *F. tularensis*), Clostridia (e.g., *C. perfriugens*), Helicobacter (e.g., *H.*

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sequence, an initiator AUG, and a mini orf (open reading frame) was positioned to mimic the native position of the S8 binding site in the *spc* polycistronic mRNA. The orf, mimicking the ribosomal protein, L5, was located upstream of S8 in the *spc* operon, followed by the S8 translational control region. A 14-base pair spacer containing a second strong SD sequence separated the two reading frames. The intervening RNA between the RNA that forms the recognition stem (the loop-stem of the stem-loop-stem structure) was truncated to a minimal UUUUU sequence, See Figure 6B. The reporter gene was constructed by standard techniques of molecular cloning.

6.2 *In Vitro* Translation Assays

The results of *in vitro* translation assays using a reporter construct that encodes an RNA binding site analog is shown in Figure 7. Recombinant S8 His-tagged S8 protein (0, 0.5, 1, 2, 3, or 4 micrograms) were added to *in vitro* translation extracts. After an initial increase in Renilla luciferase activity, Renilla luciferase activity becomes suppressed with increasing S8 concentration. The initial increase in activity may be due to a boost in translation efficiency caused by the addition of the S8 r-protein. However, after this initial increase, the sharp decline indicates that translation is being efficiently repressed by S8 binding at the S8 RNA binding site. Any test compound added to the translation reaction which restores or increase Renilla luciferase activity is a candidate for further studies.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

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4. The method of claim 1 or 2, wherein the test cell is an *E. coli* cell.

5. The method of claim 1 or 2, wherein the ribosomal protein is S8.

6. The method of claim 1 or 2, wherein the ribosomal protein is S1, S4, S7, S15, S20, L1, L4, L10 or L20.

7. The method of claim 1 or 2, wherein the reporter gene is chloramphenicol acetyl transferase, luciferase, green fluorescent protein, β-galactosidase, β-lactamase, or β-glucuronidase.

8. A method for screening for a test compound comprising:

a) contacting a test compound with a reaction mixture comprising a bacterial ribosomal protein and a reporter mRNA for a time period sufficient to allow the test compound to inhibit the activity of the bacterial ribosomal protein, wherein the reporter mRNA comprises the RNA binding site of the ribosomal protein such that translation of the reporter mRNA is repressed by the ribosomal protein; and

b) measuring the translation of the reporter mRNA, wherein a specific increase in the translation of the reporter mRNA in the test cell contacted with the test compound relative to the translation of the reporter mRNA in a test cell not contacted with the test compound, indicates that the test compound inhibits the activity of the ribosomal protein.

9. The method of claim 8 wherein the reaction mixture in step (a) comprises an excess of a recombinant ribosomal protein.

10. The method of claim 1, 2, 3, or 8 wherein the translation of the reporter mRNA is detected by a signal generated by the reporter.

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with the test compound, indicates that the test compound inhibits the binding of the ribosomal protein to the RNA.

13. A method for screening for a test compound comprising:

a) contacting a bacterial ribosomal protein with an RNA molecule comprising a binding site for the ribosomal protein and an affinity tag, in the presence of a test compound for a time period sufficient to allow binding of the ribosomal protein to the RNA;

b) contacting the RNA with a solid phase containing a binding partner of the affinity tag, for a time period sufficient to allow binding of the affinity tag on the RNA to the binding partner on the solid phase;

c) removing the ribosomal protein that are not bound to the RNA; and

d) detecting the binding of the ribosomal protein to the RNA, wherein a decrease in the binding of the ribosomal protein to the RNA contacted with the test compound relative to the binding of the ribosomal protein to the RNA not contacted with the test compound, indicates that the test compound inhibits the binding of the ribosomal protein to the RNA.

14. The method of claim 11, 12, or 13, wherein the ribosomal protein is S8.

15. The method of claim 11, 12, or 13, wherein the ribosomal protein is S4, S7, S8, S15, S17, S20, L1, L2, L3, L4, L7/12, L10, L11, L15, L20, L23 or L24.

16. A method for inhibiting the growth of bacteria comprising contacting the bacteria with a compound that inhibits the activity of the ribosomal protein identified in accordance with the methods of claim 1, 2, or 3.

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25. The method of claim 23 wherein the mammal is human.

26. The method of claim 22 further comprising adjunctively administering to the mammal a second antibacterial compound.

27. The method of claim 26 wherein the second antibacterial compound is a member of an antibiotic group selected from the group consisting of aminoglycosides, amphenicols, ansamycins, β-lactams, cephalosporins, cephamycins, monobactams, oxacephems, penicillins, lincosamides, macrolides, polypeptide antibiotics, tetracyclines, 2,4-diaminopyrimidines, nitrofurans, quinolones, sulfonamides, sulfones, oxazolidinones and glycylcycines.

28. The method of claim 23 further comprising adjunctively administering to the mammal a second antibacterial compound.

29. The method of claim 28 wherein the second antibacterial compound is a member of an antibiotic group selected from the group consisting of aminoglycosides, amphenicols, ansamycins, β-lactams, cephalosporins, cephamycins, monobactams, oxacephems, penicillins, lincosamides, macrolides, polypeptide antibiotics, tetracyclines, 2,4-diaminopyrimidines, nitrofurans, quinolones, sulfonamides, sulfones, oxazolidinones and glycylcycines.

30. A pharmaceutical composition comprising a compound that inhibits the activity of the ribosomal protein identified in accordance with the methods of claim 1, or 2, a second antibacterial compound, and a pharmaceutically acceptable carrier.

31. A pharmaceutical composition comprising a compound that inhibits the activity of the ribosomal protein identified in accordance with the methods of claim 11, 12, or 13, a second antibacterial compound, and a pharmaceutically acceptable carrier.

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SUBSTITUTE SHEET (RULE 26)

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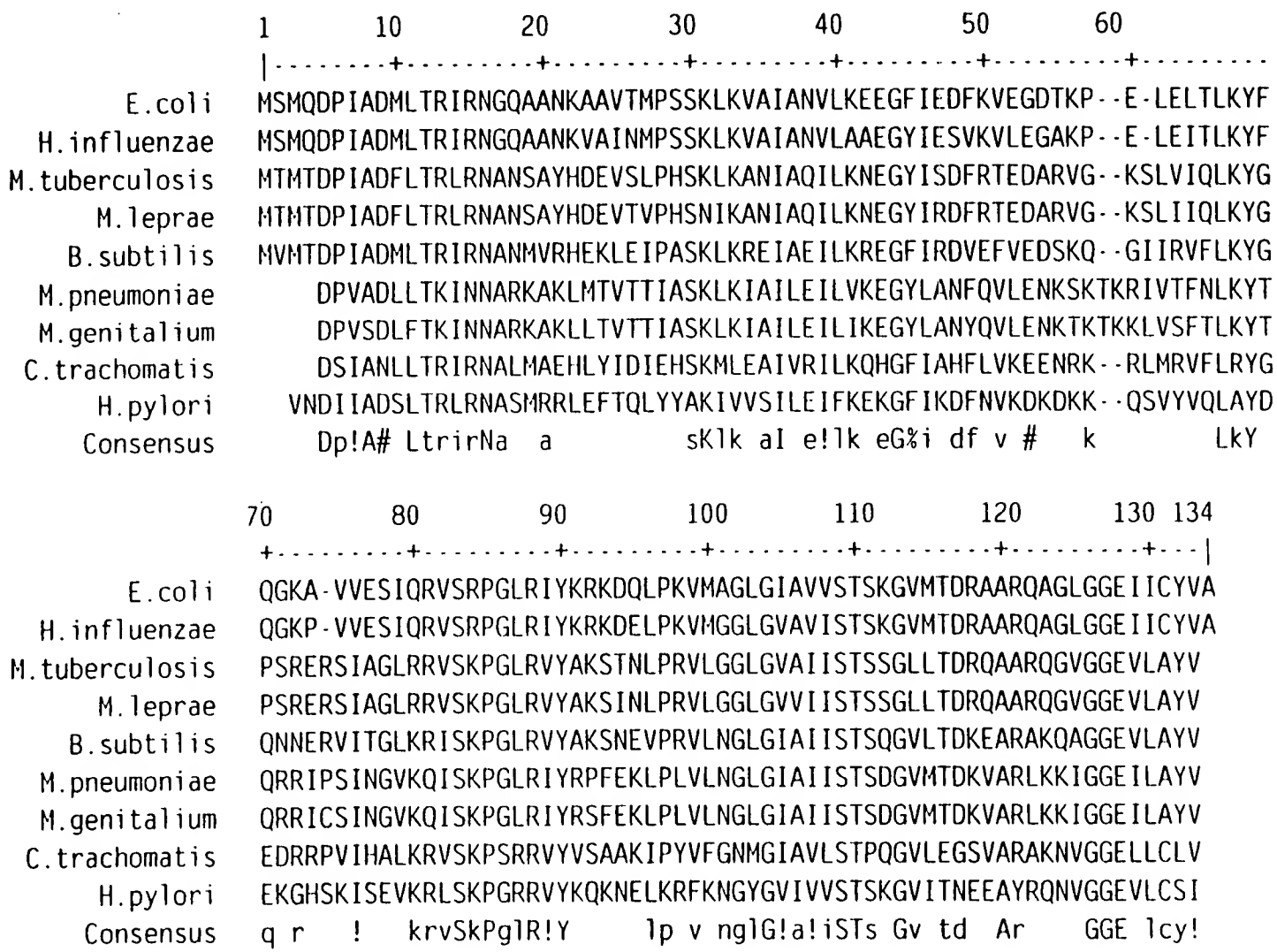
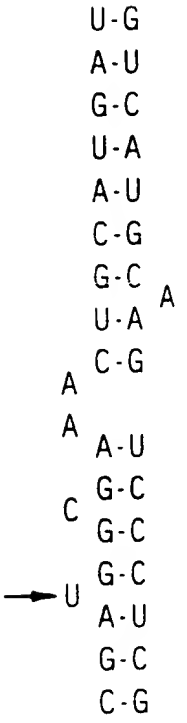
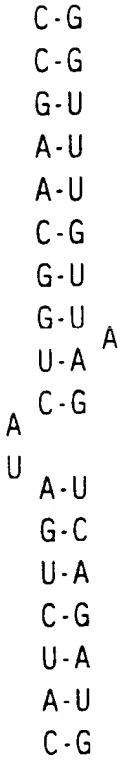


FIG.2



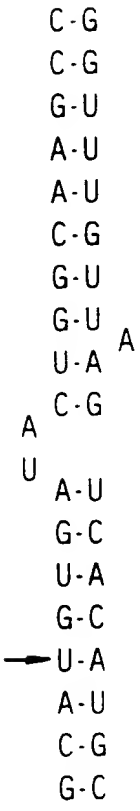
E.coli
S8/mRNA

FIG.4A



E.coli
S8/rRNA

FIG.4B



Reporter
S8/mRNA

FIG.4C

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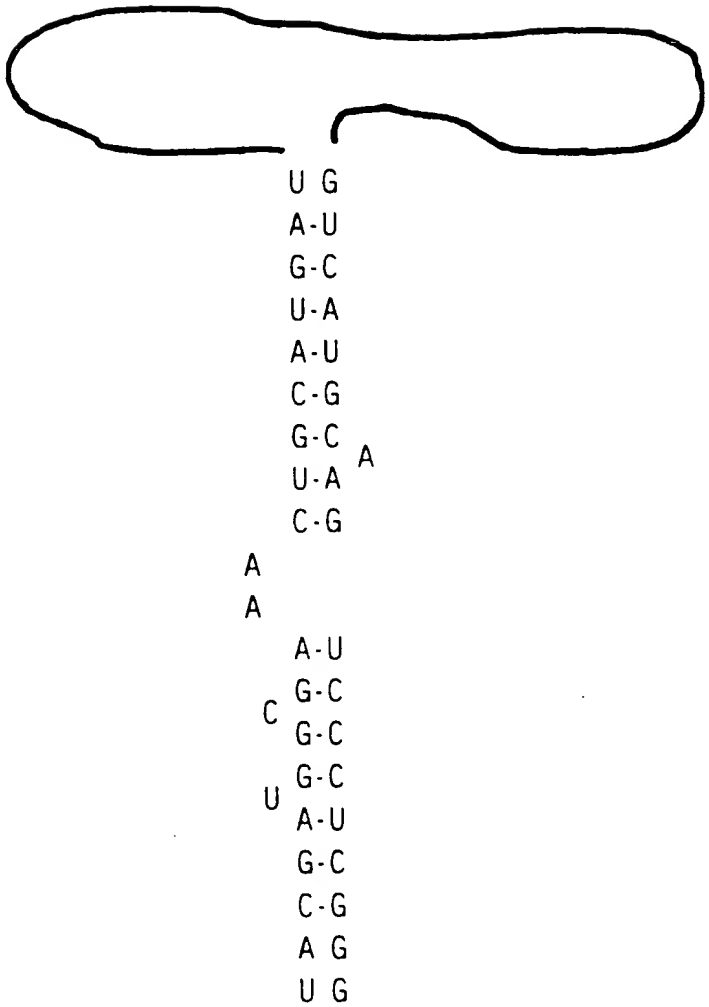

FIG.5B

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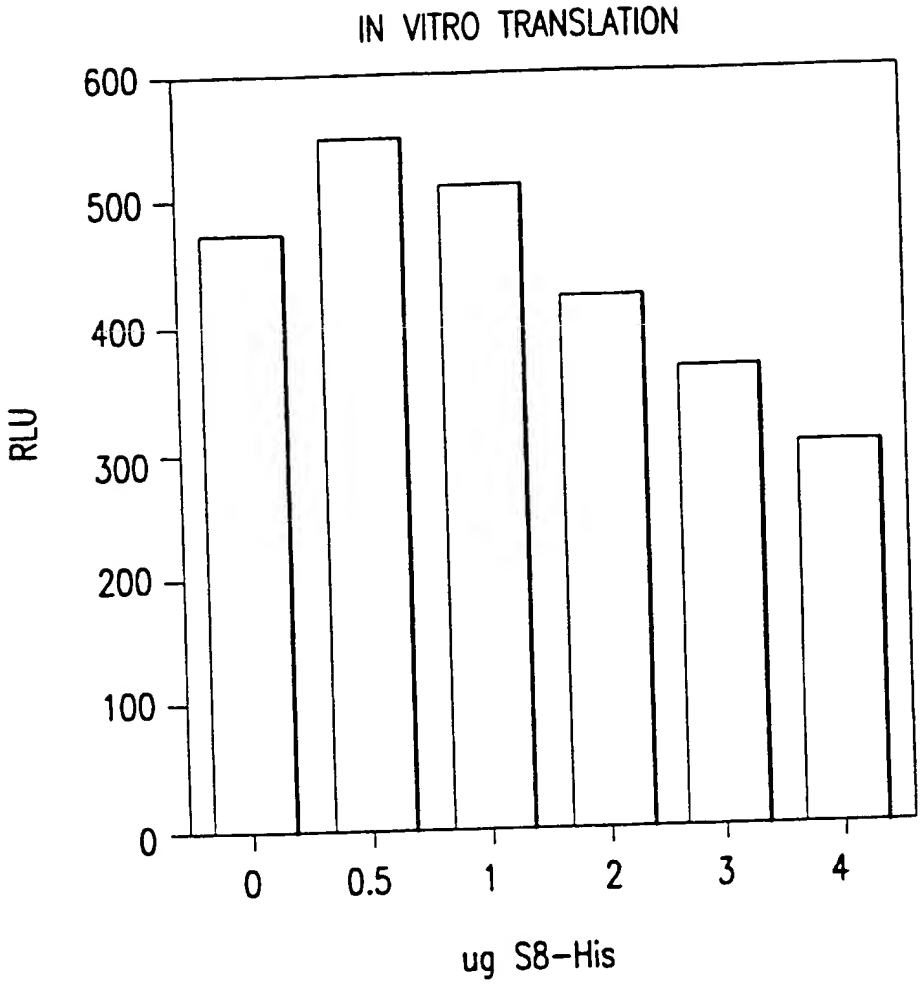

FIG.7

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/27839

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	TAKAKURA et al. Macromolecular carrier systems for targeted drug delivery: pharmacokinetic considerations on biodistribution. Pharmaceutical Research. 1996, Vol. 13, No. 6, pages 820-831, see entire document.	20-31

Form PCT/ISA/210 (continuation of second sheet) (July 1998)

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International application No.
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BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

Group I, claim(s) 1-17, drawn to a method of screening for an antibiotic.

Group II, claim(s) 18-31, drawn to pharmaceutical formulations and method of treatment.

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I is drawn to a method of screening for an antibiotic that interferes with protein synthesis. Group II is drawn to a the pharmaceutical formulation and a method treatment. The prior art discloses antibiotics that interfere with protein synthesis (Botteger E.C. Trends in Microbiology 1994 Vol 2 pages 416-421). Therefore groups I and II lack a special technical feature.

Continuation of B. FIELDS SEARCHED Item 3: WEST, MEDLINE, BIOSIS
ribosomal RNA, ribosomal protein, bacteria, antibiotics, combination drug therapy, macrolides, lincosamide, S1, S4, S7 S8, S15, S20, L1, L4, L10, L20, protein protein interaction, immobilized protein

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